AD_____

Award Number: DAMD17-01-1-0239

TITLE: Validate Mitotic Checkpoint and Kinetochore Motor Proteins in Breast Cancer Cells as Targets for the Development of Novel Anti-Mitotic Drugs

PRINCIPAL INVESTIGATOR: Timothy J. Yen, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Center
Philadelphia, Pennsylvania 19111

REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Validate Mitotic Checkpoint and Kinetochore Motor Proteins in Breast Cancer Cells as Targets for the Development of Novel Anti-Mitotic Drugs

5. FUNDING NUMBERS
DAMD17-01-1-0239

6. AUTHOR(S)
Timothy J. Yen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Center
Philadelphia, Pennsylvania 19111

E-Mail: TJ_Yen@fccc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The kinetochore is a chromosomally associated structure that is essential for accurate chromosome segregation in mitosis. Kinetochores physically link chromosomes to the spindle but also monitor these interactions through the spindle checkpoint pathway. As kinetochores function specifically in mitosis, it is a highly desirable target for development of novel anti-cancer drugs. Toward this end, we are using RNAi technology to block the expression of a select group of kinetochore proteins to determine their importance to the survival of breast cancer cells. Initial studies showed that depletion of the hBUB1 checkpoint protein from Hela cells prevented cells from arresting in mitosis in the presence of microtubule inhibitors. Loss of hBUB1 prevented other checkpoint proteins from assembling onto kinetochores. Thus, hBUB1 appears to specify the assembly of a subdomain of the kinetochore that is critical for checkpoint functions. We have expanded our studies to include the breast cancer cell lines MCF-7 and MDA-486. We will examine whether MCF7 and MDA468 cells respond in the same way as Hela cells responded to loss of hBUB1 and other kinetochore proteins. These studies may reveal that the kinetochore is a valid target for the development of novel anti-mitotic agents. In addition, it is possible that inhibition of kinetochore functions may sensitize cells to conventional chemotherapeutics such as vinblastine and paclitaxal.

14. SUBJECT TERMS	15. NUMBER OF PAGES
mitosis, microtubules, kinetochores, spindle checkpoint, breast cancer	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

Drugs that inhibit microtubule functions are one of many anti-neoplastic drugs that are used to combat breast and other cancers. Taxol and vincristine are microtubule poisons that block the proper function of microtubules that are essential for a broad spectrum of motile biological processes that include cell division, vesicle transport, cell shape, and flagella functions. For rapidly proliferating cancer cells, anti-microtubule drugs offers a highly effective means to block cell division and thus stop tumor growth. Nevertheless, these drugs block other microtubule dependent processes that adversely affect the functions of many non-dividing cells. Furthermore, there is the complication that the cancer cells can develop multi-drug resistance that makes them refractile to conventional anti-neoplastic agents (1, 2, 3). The identification of novel drugs with increased selectivity towards mitotic processes and act synergistically with existing anti-microtubule drugs should enhance and refine the modalities used to treat breast cancer patients. Our interest in the molecular and biochemical mechanisms that are central to mitosis in human cells has led to the identification of novel proteins and pathways that are suited for designing highly specific anti-mitotic drugs. The objective of this proposal is to disrupt such pathways in established breast cancer cell lines to validate them as suitable targets for developing new anti-mitotic drugs.

Body:

We proposed to manipulate two pathways that are known to be essential and operate only in mitosis of human cells to validate them as suitable targets for the development of novel anti-neoplastic agents. One pathway is specified by the kinesin-like motor protein CENP-E that is essential for aligning chromosomes at the spindle equator during mitosis. The second pathway is a checkpoint pathway that is specified by multiple proteins to ensure cells do not prematurely exit mitosis in the presence of unaligned chromosomes. We proposed four tasks to achieve our goals. We have chosen to analyze three established breast cancer lines and compare their responses to the Hela cervical carcinoma cell line, with which we have studied these two pathways extensively.

Task 1. Evaluate expression of mitotic proteins CENP-E and checkpoint proteins in established breast cancer lines.

We have conducted immunoblot analysis to determine the expression of CENP-E and the checkpoint proteins, hBUB1, hBUBR1, MAD1, MAD2 and Cdc20 in MCF7, T47D and MDA468 cells. All of these proteins were found to be expressed in these cell lines and thus confirmed that they are valid in vivo targets. Beyond the western blot data, we have also determined for CENP-E and hBUB1 kinase that they are both localized to kinetochores in MCF7 and MDA468 cells. The presence of CENP-E at kinetochores in these cell lines support our prediction that this kinesin-like protein will be critical for chromosome alignment as we have shown in Hela cells.

We have also examined the response of MCF7 and MDA468 cells to the microtubule inhibitor, nocodazole and found that this drug will delay cells in mitosis. This findings indicate that the mitotic checkpoint pathway is likely to be intact in these cancer cell lines. Thus, the various checkpoint proteins that we proposed to analyze in this project are strong candidates with which we can use to inhibit this pathway.

We are continuing with the subcellular localization of hBUBR1, MAD1, MAD2 and Cdc20 in these cell lines. Due to the fact that the morphology of these cells are not optimal for microscopy, considerable more time was required to obtain acceptable immunofluorescence images. In addition, we are also optimizing conditions that will allow us to examine these cells in real-time by videomicroscopy.

Our studies of T47D are lagging because these cells grow at only half the rate as the other cell lines. This unavoidable technical problem has prevented us from conducting all of our studies simultaneously. We therefore intend to continue to study this cell line independently of MCF7 and MDA468.

Task 2. Evaluate response of T47D, MCF-7 and MDA-MB-468 cells to inhibition of the mitotic checkpoint proteins, hBUBR1, hBUB3, cdc20 and MAD2.

As we have confirmed that these breast cancer lines express the target mitotic checkpoint proteins, we have initiated efforts to inhibit the mitotic checkpoint. We had originally proposed to accomplish this by microinjecting antibodies and overexpression of dominant negative mutants. Although we have used these methods in the past to characterize many different proteins, new advances in silencing gene expression by RNA interference (RNAi) have altered our original strategy. We have expended considerable effort to establish RNAi technology in our lab. The advantages of RNAi over microinjection and transfection of dominant negative mutants is the enhanced efficiency by which RNAi inhibits the expression of the target gene. Furthermore, the high efficiency with which the RNAi is delivered into cells reduces the problems associated with the background noise that comes from cells that were not inhibited by microinjection or expression of dominant negative mutants.

Using siRNA, we have successfully inhibited the expression of hBUB1 kinase in Hela cells. One unexpected finding was that the loss of hBUB1 prevented the assembly of MAD1, MAD2 and hBUBR1 checkpoint proteins to the kinetochore. Thus, inhibition of hBUB1 kinase may result in the inhibition of multiple checkpoint proteins. We are developing this aspect of the work. Based on our studies in Hela cells, we have begun to treat breast cancer cells with RNAi. Currently, we have treated MCF7 and MDA468 cells with hBUB1 RNAi and we are in the process of optimizing conditions to achieve the highest degree of inhibition. Once the optimal conditions for RNAi are established for each cell line, we will conduct clonogenic studies to directly examine the sensitivity of the various cell lines to loss of the mitotic checkpoint.

Task 3. Evaluate CENP-E as a target to block T47D, MCF-7 and MDA-MB-468 cells in mitosis.

As with our studies of the checkpoint pathway, we have opted to inhibit CENP-E function by RNAi technology. Using Hela cells as a positive control, we succeeded to inhibit expression of CENP-E and cells arrest in mitosis because chromosomes fail to align properly. We have initiated treatment of MCF7 and MDA468 cells with CENP-E RNAi. Once we confirm that this method can effectively inhibit CENP-E expression, we will conduct clonogenic survival studies.

Task 4. Maintaining stocks of affinity purified antibodies.

Over the past year, we have generated monoclonal antibodies to hBUB1, hBUBR1 and MAD1 proteins. The existence of monoclonal antibodies to these and other

checkpoint proteins provides us with a continuous source of high quality antibody. While the efforts to generate monoclonal antibodies are significant, we are certain that it will reduce the labor that is required to maintain stocks of polyclonal antibodies. We intend to continue to generate monoclonal antibodies to other checkpoint proteins to facilitate this research program.

Key Research Accomplishments:

- Verification of the expression of the mitotic proteins: hBUB1, hBUBR1, MAD1, MAD2, Cdc20 and CENP-E, in MCF7 and MDA468 cells.
- Established RNAi technology to facilitate the inhibition of various kinetochore proteins.
- Generated monoclonal antibodies to hBUB1, hBUBR1 and MAD1.

Reportable Outcomes:

Abstract for Era of Hope (2002 meeting).

Negotiating with Upstate Biotechnology Inc (UBI) to issue license that allows this company to market monoclonal antibodies to hBUB1 and hBUBR1 kinases.

Conclusions:

Our efforts over the last year have laid the foundation for our current efforts to directly examine the importance of the CENP-E and mitotic checkpoint pathways to the survival of breast cancer cells. We validated the expression of candidate target genes in various breast cancer lines and have initiated efforts to use the newly developed RNAi technology to efficiently inhibit gene expression in these cells. The use of RNAi technology will allow us to conduct clonogenic survival experiments that were not possible with conventional methods such as antibody microinjection and overexpression of dominant negative mutants.

If we succeed to demonstrate that breast cancer cells rely critically on CENP-E and the mitotic checkpoint functions for survival, we would then have validated that these two pathways are targets for development of anti-cancer agents. We have argued in our grant application that the ability to inhibit proteins that function specifically in mitosis should significantly enhance selectivity of chemotherapeutic agents. Thus, drugs that specifically inhibit CENP-E function should be much more selective over existing anti-microtubule drugs in killing mitotic cells. We also believe that selectivity for mitotic cells can be achieved by designing drugs that inhibit proteins that are essential for the mitotic checkpoint. In this case, disruption of the checkpoint will cause cells to divide inappropriately and create massive chromosome imbalance that is incompatible with life. It is also possible to use drugs that inhibit the checkpoint to enhance the effectiveness of conventional anti-microtubule drugs such as taxol and vincristine.

References: None

Appendices: Abstract for Era of Hope (2002 meeting).

KINETOCHORES AS TARGETS FOR CHEMOTHERAPY

S.A. Jablonski and T.J. Yen

Fox Chase Cancer Center, 7701 Burholme Avenue.,
Philadelphia, PA. USA. 19111

tj_yen@fccc.edu

The kinetochore is a macromolecular protein complex that provides two critical functions that are essential for accurate chromosome segregation in mitosis. Kinetochores contain proteins that specify the mechanical interactions between chromosomes and the spindle and are thus essential for chromosome motility. Kinetochores also contain checkpoint proteins that monitor interactions between kinetochores and microtubules to ensure that cells with even a single unaligned chromosome will not exit mitosis prematurely. Given that kinetochores function specifically in mitosis, it would appear to be a highly desirable target for development of novel anti-cancer drugs. Toward this end, we have applied recently developed technology of siRNA to selectively deplete from HeLa cells the checkpoint protein, BUB1 and the microtubule motor, CENP-E. HeLa cells depleted of BUB1 are no longer able to arrest in mitosis when its spindle is disrupted by nocodazole. Instead the checkpoint defective cells exit mitosis without dividing to be polyploid cells. Further analysis showed that the loss of BUB1 prevented several other checkpoint proteins, including CDC20, hMAD1, hMAD2 and hROD from assembling onto kinetochores. Thus, BUB1 appears to specify the assembly of a subdomain of the kinetochore that is critical for checkpoint functions. In contrast to the BUB1 studies, depletion of CENP-E from HeLa cells induces a prolonged mitotic arrest as chromosomes fail to properly align.

We have expanded our studies to include the breast cancer cell lines MCF-7 and MDA-486. Preliminary date indicate that we can deplete BUB1 and CENP-E from these cell lines using siRNA. Interestingly, the response may differ slightly between Hela cells and MDA-468 cells. Depletion of CENP-E from MDA-MB-486 cells result in fewer mitotically blocked cells as was found for Hela cells. This indicates that MDA-MB-468 cells may not have an intact spindle checkpoint. Assessment of the response of these cells to simultaneous inhibition of microtubule formation and checkpoint proteins may reveal enhanced cell killing. Ongoing studies may reveal that inhibition of spindle checkpoint pathway may sensitize cells to microtubule inhibitors such as vinblastine and paclitaxal.